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(54) Chimeric somatostatin containing protein and coding DNA, immunogenic compositions and method for increasing farm animal productivity.

(57) Chimeric somatostatins are provided comprising proteins including a protein-carrier to which a spacer

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sequence $(Sp)_n$ is coupled through ancillary linker-adaptor sequences. The spacer sequence consists of an alkaline amino acid and an amino acid ensuring a rigid β-structure, in particular Arg-Pro or Lys-Pro, coupled to a synthetic sequence encoding somatostatin-14 and a stop-codon.

DNAs coding for chimeric somatostatins comprising proteins and recombinant plasmid DNAs ensuring their expression in microorganism cells, and a method for isolation, purification and refolding of chimeric proteins are also proposed.

Immunogenic compositions are described comprising chimeric proteins as active agent to improve the productivity of farm animals.

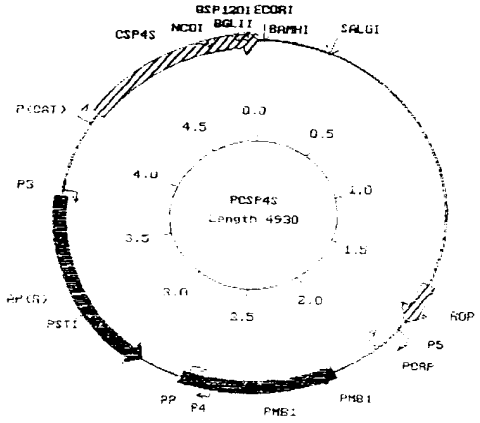


FIG. 2

The present invention relates to the field of genetic and particularly protein engineering, in particular to the preparation of chimera-derived proteins (chimeric proteins), the desired component of which for some reason cannot be obtained in a free form by microbial synthesis, and also because of its small size only has the properties of a hapten, i.e., is capable of inducing the formation of antibodies only after being combined with high-molecular carriers. In particular, the tetradecapeptide somatostatin-14 having an amino acid sequence of AGCKNFFWKTFTSC or AGCKNFFNKTFFSC relates to such a genus of oligopeptides. The use of a chimeric somatostatin-comprising protein in an immunogenic composition to increase the productivity of farm animals is also proposed.

Acceleration of the growth of farm animals at a lower cost per kilogram of weight gain is one of the main problems of stock raising. It is known that the productivity of farm animals can be increased by administration of somatotropin or some anabolic hormones or antibiotics. However, the high cost of somatotropin does not always make this method profitable, and furthermore, the use of hormonal preparations, especially anabolic ones, in the production of foodstuffs is increasingly criticized by the public. For these reasons somatotropic preparations have not yet found wide use in stock raising, and anabolic hormones are prohibited in animal husbandry. However, it is possible to increase the concentration of endogenic anabolic factors by acting on their inhibitor, i.e. somatostatin, which has good prospects of use in agriculture and in medicine (Muromtsev, G.S., et al., 1990, "Basics of agricultural biotechnology", Agropromizdat, Moscow; Keichlin S., ed., 1989, "Somatostatin, Basic and Clinical Status", Plenum Press, New York).

Somatostatin, a biologically active tetradecapeptide having the following amino acid sequence AGCKNFFWKTFTSC, is produced in the hypothalamus and the gastrointestinal tract. The sequence of somatostatin-14 is highly conservative among vertebrata, while in mammals in general it does not exist a specific species. Somatostatin has a strong inhibiting effect on a large number of hormones and functions of the organism related thereto: somatotropin, the thyrotropic hormone, insulin, glucogen, secretin, gastrin, pepsin, maletin and a number of regulatory peptides. The wide range of action of somatostatin on the factors necessary for growth and utilization of food provides a good outlook for its use as means for controlling the growth of animals, a reduction of expenditures on foodstuff, etc. .

Therefore, the autoimmune reaction to somatostatin, resulting in a reduction of the concentration of this peptide in the blood, and, as a result, the induction of anabolic factors and the acceleration of the growth of animals, is of great interest. Active or passive immunization of animals, as a result of which antisomatostatin antibodies (cf. Muromtsev et al., supra) and immunocompetent cells with antisomatostatin activity (Khodun M.V.L., Ph.D., Thesis, 1994, Moscow, Inst. Agricult. Biotechnology) appear in the blood, is used to reduce the concentration of endogenic somatostatin.

Somatostatin is a low-molecular protein-hapten; its half-life in the bloodstream is several minutes. In view of this somatostatin conjugates with various proteins are used for immunization with somatostatin. It should be underlined that this approach makes it possible to obtain ecologically pure food products, since it does not include the use of any preparations of direct hormonal effect or antibiotics, but is based on small changes in the concentration of endogenous protein anabolic factors, characteristic for elite, highly-productive animals (cf. Muromtsev et al., supra).

A large number of studies have shown that animals immunized with somatostatin have an average daily weight gain of 10 - 20 %, an appetite reduced by 9 % and an 11 % increase in the efficiency of food utilization. At the same time, improved absorption of food components and a slower passage of food through the gastrointestinal tract with sluggish peristaltics is observed. Animals immunized with somatostatin, and also their offspring, have correct proportions, and the distribution of the weight of the animals between the muscles, bones and fat is the same as in the control (cf. Muromtsev et al., supra). Immunization of gravid goats results in an increase in the weight of newly-born by 10 % and an increase in milk yield.

However, a wide use of somatostatin-14, in particular for stimulating the growth of animals by means of immunocorrection using anti-somatostatin antibodies (Muromtsev, G.S., et al., supra) is not possible because of its high price, since the main way to obtain somatostatin is by chemical synthesis (38 US dollars per mg of a somatostatin preparation, Sigma, USA, 1992); thus, it is not possible to realize this approach in practice from an economical point of view. The development of genetic engineering methods has made it possible to prepare a number of protein and peptide hormones by synthesis in the cells of microorganisms. However, it is not possible to effect the direct microbial synthesis of somatostatin using recombinant DNA technology because of its small size (only 14 amino acids; Itakura, R., et al., 1977, "Expression in E. coli of a chemically synthesized gene of the hormone sematostatin", Science, 1986, 1056-1063). Several methods for obtaining somatostatin in the form of chimeric proteins with subsequent specific cleavage of the product of interest have been described (Itakura, R., et al., 1977, Russian patent application No. 4921158/13 of

March 26, 1991).

The first research in respect of obtaining somatostatin-14 by use of genetic engineering technology was conducted in 1977 by Itakura. The authors constructed a hybrid gene on the basis of E. coli, β-galactosidase, in the C'-end region of which a chemically synthesized sequence of somatostatin was engineered. Later, effective producers of chimera derived proteins were created on the basis of that process with a sequence of somatostatin introduced into chimeric trpE, trpD and recA E. coli genes. The level of expression of chimeric proteins reached 15 - 30 % of the total amount of proteins, while the output of somatostatin did meet the requirements of industrial production (Itakura, R., et al.)

A recombinant plasmid DNA encoding somatostatin and a strain of E. coli producing somatostatin are disclosed in Russian patent application No. 4921158/13 with priority of March 26, 1991. This plasmid determines the constitutive synthesis of a hybrid protein of chloramphenicol acetyl transferase-somatostatin-14 under the control of its own promoter in cells of E. coli MKD3207 with a reduction of the level of degradation of anomalous proteins. However, the genetic engineering constructs described above allow only the preparation of chimeric proteins, the immunogenic activity of which with respect to somatostatin is extremely low, and therefore these proteins are not yet being used in agriculture or medicine.

It is the object of the present invention to provide chimera-based immunogenic proteins including somatostatin-14, corresponding recombinant DNA and expression plasmids, methods for preparing these proteins and the respective plasmids, transgenic cells for use in these methods, immunogenic compositions and methods for increasing the productivity of farm animals.

The above object is achieved according to the independent claims. The dependent claims relate to preferred embodiments of the concept of the invention.

The chimeric immunogenic polypeptides or proteins of the present invention include somatostatin-14, coupled to a protein carrier through a spacer $(Sp)_n$ ensuring the positioning of the somatostatin on the surface of the carrier. The number of monomeric proteins of the spacer (n) is preferably from 1 to 8, while the spacer (Sp) includes a sequence of amino acids comprising or consisting of an alkaline amino acid and an amino acid ensuring a rigid β-structure, in particular, Lys-Pro or Arg-Pro. The invention further relates to recombinant DNA molecules coding for the aforementioned chimeric proteins, to a process for preparing the immunogenic chimeric proteins, and also to bacterial strains, preferably of Escherichia coli, transformed with the aforementioned DNAs and producing these proteins. In accordance with the invention, the chimeric protein is the active ingredient of immunogenic compositions for improving the productivity of farm animals. The proposed system for immunization of animals with a composition including the aforementioned chimeric protein results in a stable increase in productivity, which is the result of the specific effect of anti-somatostatin immunization and the unspecific effect of stimulation, which effect is caused by the immunization procedure itself.

Brief Description of the Drawings

Fig. 1 is a schematic drawing of recombinant DNA plasmids coding for chimeric somatostatin-comprising proteins, $pC(Sp)_nS$.

Fig. 2 shows a physical map of a pCsP4S plasmid.

Fig. 3 shows a physical map of a pTaCAP4S plasmid.

Fig. 4 shows a physical map of a pT5CAP4S plasmid.

Fig. 5 shows a physical map of a pT5DAP4S plasmid.

Detailed Description of the Invention

Engineering a recombinant molecules of DNA coding for a chimeric protein having immunogenic activity provides for the exposition of an antigenic determinant of interest on the surface of protein carriers. This is achieved by joining a chemically synthesized nucleotide sequence coding for an amino acid spacer $(Sp)_n$ connected to an area of a DNA coding for an antigenic determinant to the 3'-terminus of a gene of a protein carrier through an adaptor.

The step of engineering a chimeric protein with a spacer and its subsequent synthesis is prospective to obtain antibodies to antigens of relatively small peptides, the microbial synthesis of which in a free form is difficult, while chemical synthesis is expensive: somatostatin, an epidermal growth factor, eukaryotic antibiotics and some natural and synthetic peptides. The invention relates to an increase in the meat and milk productivity of farm animals using recombinant somatostatin-comprising proteins. New immunogenic chimeric proteins are provided including an amino acid spacer (Sp) comprising or consisting of an alkaline amino acid and an amino acid ensuring a rigid β-structure, in particular lysine and proline or arginine and

proline, resulting in localization of somatostatin on the surface of a carrier, which ensures its high immunogenicity.

Preferably the number of monomeric blocks (n) in the spacer is from 1 to 8, depending on the structure of the protein carrier and localization of the somatostatin.

Various proteins, including bacterial chloramphenicol acetyl transferase (CAT) with different kinds of deletions, β-galactosidases, dihydrofolate reductases, hydrophobic synthetic polypeptides, etc., can be used as carriers for somatostatin.

It is preferable that inactive chloramphenicol acetyl transferase without 10 C-terminal amino acids be used for large-scale synthesis of chimeric somatostatin-comprising proteins. Chimeric somatostatin-comprising protein with the aforementioned chloramphenicol acetyl transferase, obtained as a result of microbial synthesis, turned out to be hydrophobic. Both constitutive, in particular, its own promoter of chloramphenicol acetyl transferase in the construction $pC(Sp)_nS$, and inducible promoters (Tac, Lac, Trp, T5) in some other plasmids (see Figs. 2-5) can be used to express a chimeric somatostatin-comprising protein. With small-scale microbial synthesis of a chimeric protein in fermenters of up to 100 l wherein chloramphenicol acetyl transferase (CAT) is used, it is preferable that the CAT promoter itself be used as the carrier. With large-scale synthesis in fermenters of 0.05 to 1 m^3 and larger, and also when other proteins are used, it is preferable to use inducible promoters. It is most preferable to use a T5-Lac promoter with IPTC as the inductor.

Recombinant plasmid DNAs ensuring expression of chimeric somatostatin-comprising proteins under the control of CAT, Trp, T5 promoters with CAT and DHFR proteins in E. coli cells used as the carriers are shown in Figs. 1-5.

It should be noted that the aforementioned recombinant plasmid DNAs are only illustrations of the invention. The successful expression of chimeric DNA constructs according to the invention may be achieved, as stated above, with the use of other promoters and protein carriers in suitable cells of microorganisms.

A $pC(Sp)_nS$ series of plasmids for expression of a chimeric somatostatin-comprising protein under the control of its own CAT promoter has been constructed using a CAT protein without the 10 C-terminal amino acids as the carrier, wherein n is 1, 2, 4 and 8 (see examples 1, 2 and 3).

pTRCAP4S (Fig. 3) comprising the promoter of the tryptophane operon, pTACAP4S (Fig. 1) comprising the TAC-promoter, and pT5CAP4S with a phage T5 promoter (Fig. 4, example 6) are realizations of constructs in which expressions of DNA coding for chimeric immunogenic somatostatin-comprising proteins are under the control of inducible promoters.

One of the variants of the constructs in which proteins distinct from chloramphenicol transferase are used as carriers is the recombinant plasmid DNA pT5DAP4S (Fig. 5, example 7) comprising a gene of dihydrofolate reductase as the gene of the protein carrier.

The method for preparing chimeric immunogenic somatostatin-comprising proteins includes cultivating cells of microorganisms transformed by recombinant plasmids of DNA comprising sequences coding for the aforementioned proteins under conditions allowing their expression, and subsequent purification of the product of interest. Any suitable cells of microorganisms can be used as host cells, while any known plasmid systems suitable for expression in the selected host cells can be used as the expression vectors.

Purification of the product of interest is effected in accordance with known technology, including, for example, lysozyme lysis, differential centrifugation of inclusion bodies, gel permeation chromatography, etc. Refolding procedure is conducted in guanidine chloride at alkaline pH values followed by dialysis and lyophilization. The yield is about 0.4 - 9 of the protein per litre of bacterial suspension. A person skilled in the art can easily select suitable methods for purification of the product of interest depending on the specific conditions.

In the preferable embodiments of the process for microbial synthesis of chimeric somatostatin-containing proteins according to the invention, DNA constructs were used, as a result of the expression of which hydrophobic polypeptides were synthesized since hydrophilic chimeras were subjected to hydrolysis with bacterial proteases. Obtaining the product of interest in the form of hydrophobic inclusion bodies, on the one hand, isolates and protects the recombinant proteins from the effect of the protease, and on the other hand, prevents possible undesirable effects of an excessive amount of foreign proteins on the metabolism of the producing cells. The use of bacterial strains as producers, deficient in respect of proteases, in particular E. coli lon^- mutants, also contributes to an increase in the output of chimeric proteins.

In accordance with the invention chimeric proteins produced by cells transformed with DNA recombinant plasmids interact with the antibodies to somatostatin-14, and, when animals are immunized, induce the synthesis of somatostatin-specific antibodies and immunocompetent cells specific to somatostatin. The

amount of anti-somatostatin antibodies in the serum of immunized animals is determined by means of radioimmunoassay.

The contents of immunocompetent cells are determined by the method of biochemoluminescence after sensibilization with somatostatin (Khodun M.V.L., Ph.D., Thesis, "Study of physiochemical immunological and biological properties of somatostatin-14 containing recombinant proteins and evaluating their possible use for stimulation of productivity of domestic animals", Moscow, Inst. Agricultural Biotechnology, 1994).

The molekular weight of the chimeric protein, determined by the method of electrophoresis in polyacrylamide gel under denaturating conditions, is 28 kDa.

In accordance with the invention the immunogenic compositions for animals comprise a chimeric protein in an effective amount together with usual carriers, excipients and/or adjuvants, for example an emulgator or an excipient suitable for animals, such as for example a solution of vitamins in vegetable oil, adjuvants, heated beeswax, etc. Preferably, an incomplete standard Freund's adjuvant is used. The protein carrier is not specifically limited. In particular it may be chloramphenicol acetyl transferase without the 10 C-terminal amino acids, or dihydrofolate reductase. In the amino acid spacer $(Sp)_n$, n is preferably from 1 to 8, depending on the structure of the protein carrier. The somatostatin-14 preferably has the sequence AGCKNFFWKTFTSC.

In accordance with a preferred embodiment, the weight ratio of the purified chimeric somatostatin-comprising protein to the incomplete Freund's adjuvant is from 1:0.05 to 1:10, preferably 1:0.5, and most preferably 1:1 or 1:2.

The purpose of the compositions described above is to increase the productivity of farm animals. The process of increasing the productivity of farm animals comprises administering to the animals a preparation comprising a chimeric immunogenic protein in accordance with the invention, preferably by injection. The preparation is injected intramuscularly or subcutaneously, preferably 3 to 5 times. In accordance with a preferred embodiment, the first three injections are given at intervals of 1o to 14 days, and then two booster injections are additionally given at two-month intervals.

In special embodiments of the claimed process immunization of gravid dams is carried out approximately 4o to 50 days before bringing forth young so as to prevent a lag phase in the induction of anti-somatostatin antibodies and immunocompetent cells targeted at somatostatin in the new-born when it is immunized. The doses depend on the kind of animal and the mode of immunization, the preferable dose being 40 to 60 µg/kg of weight of the animal.

It should be underlined that the increase in meat and milk productivities of all animals tested caused by anti-somatostatin treatment is not accompanied by an increase of feed consumption. In some cases anti-somatostatin immunocorrection is accompanied by improving the quality of animal products (decrease of fat content).

Examples will now be given illustrating but not limiting the present invention.

Example I

Assembly and molecular cloning of the nucleotide sequence of a spacer (Sp) Arg-Pro or Lys-Pro.

In the case of the spacer Arg-Pro oligonucleotides (2 sequences GATCTATGC and AATTGCATA forming an adaptor, and 2 sequences GATCTGGGCCCCGGCCGG and AATTCCGGCCGGGGCCCA forming a spacer) are synthesized by the amidic method in a solution in a PS 200 Cruachem synthesizer (England). Each of the chains of synthesized nucleotides is phosphorylated separately. The reaction is carried out in 10 mM of a TrisHCl buffer, pH 7.5, comprising 10 mM of $MgCl_2$, 50 mM of dithiothreitol, 1 mM of ATF and 100 pM of oligonucleotide, and 1 unit of T4 phage polynucleotide kinase during one hour at 37 °C. After the reaction is completed, the enzyme is inactivated by heating for 10 min at 65 °C.

In order to obtain a hybrid protein comprising a Lys-Pro spacer, a fragment coding for Arg-Pro is cut from a plasmid $PC(Sp)_n4S$ at BglII-EcoRI sites, and a sequence of 36 nucleotides

GATCCGGAAAACCGAAACCGAAACCGAAACCCGGGGGCCTTTGGCTTTGGCTTT
GGCTTTGGGCCCCTTAA

coding for the spacer $(Lys\text{-}Pro)_4$ is inserted into its place. The length of the aforementioned sequence accordingly changed with the degrees of polymerization, $n > 4$ or $n < 4$.

A recombinant plasmid pCCs of 4920 bp (Russian patent application No. 4921168/13 of March 26, 1991) is used to clone the spacer sequence Sp. This plasmid comprises a fragment of vector pBR325 of 4860 bp with a gene of β-lactamase and a part of a modified gene of chloramphenicol acetyl transferase (CAT), to its 3'-terminus a synthetic gene of somatostatin-14 is linked through a synthetic linker with the EcoRI and flanked at the 5'-terminus with the nucleotide sequence GG. This plasmid determines the constitutive synthesis of a hybrid protein CAT-somatostatin-14 under the control of its own CAT promoter in cells of E. coli. Cloning of Sp is carried out in two stages. At first a derivative pCCs is obtained in which the site of restriction endonuclease EcoRI is replaced by the site BglII.

1A. Molecular cloning of a BglII-EcoRI adaptor.

1 μg of the plasmid pCCs is incubated with restriction endonuclease EcoRI in a buffer of 50 mM of Tris-HCl, pH 7.5, 100 mM of NaCl, 7 mM of $MgCl_2$, 7 mM of β-mercaptoethanol at 36 °C for 1 hour. 50 mM of each phosphorylated oligonucleotide of the adaptor is added to 1 μg of linearized plasmid pCCs. Combining the mixtures of oligonucleotides and plasmid DNA is carried out in a buffer for kinase comprising 1 unit of the T4 phage ligase. Incubation is carried out at 12 °C during 16 hours. The ligated mixture of DNA and oligonucleotides is introduced by transformation into cells of E. coli HB101. Transformation is carried out using frozen cells; 5 fresh colonies are dispersed on a shaker in 1 ml of a SOB medium (2 % trypton, 0.5 yeast extract, 10 mM of NaCl, 10 mM of $MgCl_2$, 10 mM of $MgSO_4$). 500 μl of a suspension of cells are introduced into 10 ml of a liquid SOB culture medium and are grown at 37 °C to a cell density of 7×10^7 cells/ml. The cells are cooled on ice and centrifuged at 800 g during 15 minutes at 4 °C. The supernatant is thoroughly removed, and the deposit is suspended in 3.3 ml of a buffer with rubidium chloride (100 mM of RbCl, 50 mM of $MgCl_2$, 30 mM of KOAc, 10 mM of $CaCl_2$, 10 mM of glycerin, pH 6.8). The cells are kept on ice for 2 hours, and then centrifuged; the supernatant is thoroughly removed. It is resuspended in 800 μl of buffer comprising 10 mM of MOPS, 10 mM of RbCl, 75 mM of $CaCl_2$ and 15 % of glycerol, and incubated for 15 minutes on ice. After that aliquots of the final suspension are frozen in liquid nitrogen and used for transformation.

200 μl of the prepared suspension of cells are mixed with 5 μl of a solution of the ligated mixture and incubated for 30 minutes on ice, then for 90 seconds at 42 °C, then again on ice for 2 minutes. 800 μl of the SOB medium are added and incubated with mild shaking at 37 °C during 60 minutes. A small portion of the suspension of cells is seeded on agar plates, prepared on an SOB medium, comprising 1 % of bactoagar and 50 μg/ml of ampicillin. 12 clones grown in the plate with ampicillin are arbitrarily selected, and the plasmid DNA is separated using the alkaline method. After treatment of these plasmids with restriction endonucleases PstI and BglII and agar gel electrophoresis, the plasmid of the desired construction is selected.

1B. Cloning an Sp sequence

An initial plasmid pCCS and its modified derivative pCCS-BglII are used to clone an Sp sequence (Arg-Pro or Lys-Pro). 3 μg of the plasmid pCCS are incubated with restriction endonucleases PstI and EcoRI, while plasmids pCCS-BglII - with restriction endonucleases PstI and BglII in a buffer under conditions as in example 1a. The fragments are separated by electrophoresis in an 0.8 % agar gel. In the first case a large fragment (3296 bp) is cut from the gel and extracted from the gel with five volumes of 1.5 M of NaCl. The supernatant obtained after centrifugating the suspension is treated with chloroform; the DNA is precipitated with three volumes of ethyl alcohol. The DNA is dissolved in 10 μl of distilled water. In the second case a fragment of lower molecular weight (1175 bp) is cut from the gel and treated in a similar manner. 50 pM of each phosphorylated oligonucleotide SS is added to 5 μl of the DNA preparation of each fragment. Ligation of the mixture of oligonucleotides and DNA fragments is carried out as in Example 1a. Transformation of the ligated mixture and selection of the clones are carried out as in Example 1a. An analysis of the sequence of the nucleotide insert was carried out initially with restriction mapping for the presence of restriction sites Bsp1201 and Eco521, and then the analysis of the nucleotide sequence was carried out by the Maxam-Gilbert method.

Example 2

Polymerization of Sp

The nucleotide sequence Sp (Arg-Pro) includes restriction sites Bsp1201 and Eco521, the distinction of which is the presence of identical "sticky" ends with different flanking nucleotides in the first and sixth positions. Such an organization of sites makes it possible to conduct polymerization of the Sp, resulting in its size being increased. To do this 3 μg of the plasmid pCsPs in the first case are incubated with restriction endonucleases PstI and Bsp1201, and in the second case with restriction endonucleases PstI and Eco521. A large fragment (3302 bp) was obtained from the products of the first hydrolysis, and a fragment of lower molecular weight (1616 bp) from the products of the second hydrolysis. The procedures of elution, ligation, transformation, and selection of clones were carried out as in Example 1a. The DNA from the obtained clones was first analyzed by restriction mapping with endonucleases NcoI-Bsp1201 and NcoI-Eco521. The clones did comprise dimer Sp and additional 6 nucleotide pairs as compared with the initial clones. The plasmid was designated as $pC(Sp)_2S$. The polymerisation procedure was carried out two more times; as a result, clones were obtained with tetra- and octomers of Sp designated as $pC(Sp)_4S$ and $pC(Sp)_8S$. These constructs are designated in general by (Sp)n. In the examples described below plasmids with n = 4, i.e., $pC(Sp)_4S$, were used in the majority of cases, although both $pC(Sp)_2$ and $pC(Sp)_8$ were also used. Polymerisation of the spacer Lys-Pro was not carried out by this method. The necessary degree of polymerization was attained by chemical synthesis of the longer oligonucleotide (see Example 1).

Example 3

Use of recombinant plasmids of the $pc(Sp)_nS$ series to obtain producing E. coli strains.

Plasmids $pC(SP)_nS$ are introduced by transformation into E. coli strain MKD3207 by the method described in Example 1a, and strains producing the hybride proteins are obtained.

The strain MKD3207 is characterized by the following features:

Culture and morphological properties

The strain MKD3207 (derived from Escherichia coli K12) is composed of gram-negative, slightly-mobile rods, under unfavorable conditions forming filaments. The strain grows well in the temperature range of 30 to 42 °C on rich LB type mediums, and also on synthetic mediums with additives compensating auxotrophic mutation. On rich mediums the strain forms smooth colonies with even edges which in time mucify, this being due to 1on-mutation. Mucifying colonies does not occur at an incubation temperature of 40 to 42 °C. The colonies are always mucific when grown on a synthetic medium with additives.

Genetic and physiological-biochemical features

The strain MKD3207 has the following genetic markers: F^-, lacY, SupE, ga16, xy14, mal Al, arcH, Ris', Lon^-, apr 24, rpl. It is stable against streptomycin, and does not ferment lactose, galactose, xylose and maltose. The strain grows on a synthetic medium with additives of glucose, arginine and histidine. The strain MKD3207 comprising the plasmid $pC(Sp)_nS$ acquires resistance to ampicillin.

An analysis of the expression of genes coding for somatostatin as a part of hybrid proteins is conducted in cells of E. coli MKD3207. The hybrid genes in the expressing vectors harbouring E. coli MKD3207 cells determine the constitutive synthesis under the control of its own CAT promoter. The cells E. coli MKD3207, transformed by plasmids $pC(Sp)_1S$, $pC(Sp)_2S$, $pC(SP)_4S$ and $pC(Sp)_8S$, are grown in a ILB medium comprising ampicillin (50 μg/ml) to a density of OD_{550} 2.0-2.5 at 37 °C during 18 hours. The original plasmid pCCS coding for chimeric protein CAT-somatostatin under the control of its own CAT promoter P_{cat} is used as the control. A sediment of cells obtained from 1.5 ml of cell culture by centrifuging is then suspended in 200 μl of a buffer solution comprising 50 mM of Tris-HCl, pH 6.8, 10 % glycerol, 2 % SDS and 2 % β-mercaptoethanol. The suspension is kept boiling for 5 minutes and analyzed by means of SDS-PAGE electrophoresis in 15 % SDS-PAG. The results show the presence of a dominating band having a molecular weight of 26.5 kDa for a chimeric protein with monomeric and dimeric copies of the spacer, 28 kDa for a tetrameric spacer and 30 kDa for an octomeric spacer sequence. The level of expression of hybrid proteins with monomeric, dimeric and tetrameric spacer sequeces is approximately equal and is 30 % of the total bacterial proteins, and with an octomeric sequence lower by 5 %. The strain Escherichia coli

MKD3207, transformed by plasmid pC(Sp)$_4$S was deposited in the All-Russian Collection of Cultures of Industrial Microorganisms under accession number B-6519.

Example 4

Preparation of a hybrid protein with a somatostatin sequence.

Cells of E. coli MKD 3207 transformed by plasmid pC(Sp)$_4$S are cultivated in a medium 1LB as described in Example 3 in a fermenter to a density OD_{550} of 4.0 to 5.0. The cells are deposited by centrifuging at 5000 g for 10 minutes. The sediment of the cells is suspended in 50 mM of Tris-HCl, pH 8.0, comprising 50 mM of NaCl, 11 mM of EDTA calculated on the basis of 38 ml of a buffer for a biomass of one liter of cell culture. After suspending the cells lysozyme is added to a final concentration of 100 µg/ml, and Triton-X100 to a concentration of 0.1 %; the suspension is then incubated on ice. The cells are disrupted by ultrasound. The sediment, including hydrophobic hybrid protein in the form of inclusion bodies, collected by centrifuging at 12000 g and 4 °C for 10 minutes, is washed twice with a Triton containing buffer, and centrifuged and resuspended in the original buffer without Triton. Aliquots are removed and analyzed by SDS-PAGE using 15 % SDS-PAG. As a result of this purification procedure a preparation of hybrid protein is obtained having a purification degree of more than 90 % of the total proteins deposited.

Purification of chimeric proteins with other carriers is carried out according to the method described above.

This method of separation and purification of the hybrid protein has been adapted for subsequent use of the product as a preparation for stimulation in animal husbandry.

For immunological analysis a preparation of a protein obtained using the method described in Example 4 is dissolved in 6 M of guanidine chloride, then dialyzed against an 8 M urea solution in 10 mM of a carbonate buffer, pH 11, for two hours, and diluting the dialyzed solution with 10 mM carbonate buffer, pH 11, twice, every two hours, bringing the concentration of urea to 1 M. Then dialysis is conducted against 10 mM of a phosphate buffer, pH 7.6, during 12 hours. The resulting solution is used as an antigen (adding to a reaction mixture in an excessive amount) in a competitive RIA, conducted using a commercial kit ("Incstar", USA). The preparation inhibits the binding of I^{125}-labelled somatostatin with specific anti-somatostatin antibodies (Table 1).

Table 1

Immunological analysis of protein preparations		
	Sample	Binding (%)
1	B_o (sample without preparation being studied)	100
2	Background (sample without specific antibodies)	16
3	Control (sample with a carrier protein-chloramphenicol acetyl transferase)	98
4	Experimental (sample with CAT-SS-somatostatin)	17

Example 5

Obtaining a preparation for immunization

To purified protein isolated from hydrophobic inclusion bodies and dissolved in 0.2 M Tris-HCl buffer, pH 8.0, comprising 6M of guanidine chloride and 2 mM of EDTA, a 50-times molar excess of β-mercaptoethanol is added per 1 mole of S-S groups of chimeric protein, and the solution is rapidly diluted in a 10-fold volume of buffer without guanidine chloride. The sedimented hybrid protein is separated by centrifuging for 15 minutes at 12000 g and 4 °C. Then the deposit is suspended in sterile distilled water and centrifugated, as described above, repeating the treatment twice. The final product is lyophilized for storage. Just before use the lyophilized preparation is re-suspended in a minimum volume of 10 mM of a phosphate buffer, pH 7.0, then an equal volume of Freund's incomplete adjuvant is added, and the aqueous-oily suspension is homogenated under short sonification. Immunization is conducted by intramuscular or subcutaneous injection of the suspension in the region of the neck or shoulder blade with a dose of 50 µg of chimeric protein per kg of live weight. The injection is repeated three times at two-week

intervals. Then, depending on the length of the incubation period, 1 to 2 more booster immunizations are given so that the productivity of the animals is increased to the desired level as they grow older.

Example 6

Engineering inducible producers of a chimeric protein with a somatostatin sequence.

Constructs similar to $Pc(Sp)_4S$ were prepared for use for a number of purposes including large-scale industrial synthesis of anti-somatostatin chimeric proteins. To this end the hybrid gene CAP-4S (CAT-210 amino acids, spacer Arg-Pro or Lys-Pro) and somatostatin was put under the control of a tryptophane operon (construct pTRCAP4S), a phage T5 promoter (construct pT5CAP-4S) and a TAC-promoter (construct pTACAP4S). The number of monomeric blocks of the spacer in the plasmid pTACAP4S is as in the plasmid pC(Sp)4S equal to four. A plasmid having a size of 4994 bp comprises a Scai-BamHI fragment of a plasmid vector pBR325 with a size of 4910 bp, including a part of a gene of tetracycline resistance with a BamHI site at the C-end, a gene of ampicillin resistance, a promoter region TAC of a plasmid pDR540, a part of a gene of chloramphenicol acetyl transferase with a half-site of ScaI at the 3'-end and an eliminated site of EcoRI, a SmaI-EcoRI fragment of a linker comprising a site of EcoRI and flanked with the 5'-end of a nucleotide sequence GGG of a half-site SmaI for joining to the 3'-end of a gene of chloramphenicol acetyl transferase with site ScaI, a EcoRI*-BglII fragment of an adaptor comprising a site of EcoRI*-BglII for connection to the 9 bp sequence of a spacer, a BglII-EcoRI spacer sequence of size 36 bp and a EcoRI-BamHI fragment of a synthetic gene of somatostatin with a "stop"-codon of size 54 bp. Genetic marker of this plasmid is a resistance to ampicillin. All three aforementioned constructs synthesized are authentic chimeric proteins differing only in their transcription regulatory elements. The procedure of purifying and refolding was essentially similar to the above described one. The properties of proteins and the physiological effects caused thereby in the case of immunization of animals were also identical.

All three constructs are identical to $pC(Sp)_4S$ with the exception of the promoter region.

A strain comprising a plasmid pTACAP4S does not synthesize chimeric protein with a somatostatin sequence when grown on poor and enriched media. Induction of protein synthesis is effected by the addition of an isopropylthiogalactoside (IPTG) inductor. The synthesis of a protein in a strain comprising plasmid pT5CAP4S is induced in a similar manner. The synthesis of protein in a strain comprising plasmid pTRCAP4S is induced by β-indolylacrylic acid. The amount of protein synthesized with inducible strains reached 40 % of the total bacterial protein.

The molecular weight of the chimeric protein determined by the electrophoretic mobility in a PA gel under denaturating conditions is 28 kDa as in the case of pC(Sp)4S.

The chimeric protein interacts with antibodies against somatostatin-14 and in the case of immunization of animals induces the immuno-competent cells and somatostatin-specific antibodies.

Example 7

Engineering of a recombinant plasmid with a gene of mice dihydrofolate reductase as the carrier.

A construct pT5DAP4S (Fig. 5) was prepared to study the physiological effects caused by a protein carrier (see below), wherein modified mice dihydrofolate reductase gene was used for the protein carrier. The region coding for the spacer Arg-Pro and somatostatin was used without changes. A hybrid gene DAP4S was placed under the control of a phage promoter. The number of monomeric blocks of the spacer is, as in the plasmid $pC(Sp)_4S$, equal to four. Plasmids having a size of 3553 bp comprise a fragment AatII-NdeI of a plasmid vector PBR322 having a size of 1992 bp, including a gene stable to ampicillin, a promoter region T5, a part of a gene for dihydrofolate reductase from the plasmid vector pQE16 with a half-site of restriction BglII at the 3'-end for connection with a sequence of a spacer, a BglII-EcoRI spacer sequence with a size of 36 bp and a EcoRI-BamHI fragment of synthetic gene of somatostatin with a "stop"-codon having a size of 54 bp, a gene of chloramphenicol acetyl transferase. The genetic marker is resistant to ampicillin. The synthesized chimeric protein had similar physico-chemical properties. The procedure for purifying and preparing the preparation for immunization corresponded to a standard process. The physiological effects caused by the introduction of hybrid protein with dihydrofolate reductase were identical to those already described.

A strain comprising a plasmid pT5DAP4S does not synthesize a chimeric protein with a somatostatin sequence when being grown on poor and enriched media. Induction of protein synthesis is effected when isopropylthiogalactoside (IPTG) is added as inducer. The amount of protein synthesized with inducible

strains reaches 40 % of the total bacterial protein.

The molecular weight of the chimeric protein determined by the electrophoretic mobility (PAGE) in a PA gel under denaturating conditions is 26 kDa.

The chimeric protein interacts with antibodies against somatostatin-14, and in the case of immunization of animals induces the synthesis of immuno-competent cells and somatostatin-specific antibodies.

Example 8

Use of chimeric somatostatin-comprising proteins to increase productivity.

8a. Milk yield of milkers

The preparation is injected into gravid heifers of black breed, 24-25 months old, approximately 50 days before calving, the time of calving being determined by rectal test. The dose is 50 g per kg of live weight; the injections are made in the region of the neck or shoulder blade and repeated three times at two-week intervals. Under these conditions, as shown above, anti-somatostatin immunocompetent cells and corresponding antibodies appear in the blood, while no toxicity of the preparation can be detected in the immunized animals, and no disturbances in the reproducing functions (abortions, still-births, abnormities, etc.) are observed. Somatostatin-comprising proteins with chloramphenicol acetyl transferase (CAT) and dihydrofolate reductase (DHFR) as carriers were used in the experiment.

In order to conduct analysis of the induction of specific antibodies against somatostatin, caused by immunization with the preparation, blood is taken from gravid cows and cows who have calved 7 days after the last injection. The plasma is obtained and is studied by radioimmunoassay using a known procedure and a commercial kit ("Incstar", USA). Specific binding of somatostatin is observed in preparations of plasma of the animals in the group being studied (Table 2).

Table 2

Induction of specific antibodies against somatostatin		
	Sample	Binding (%)
1	B_o (sample without preparation being studied)	100.00
2	Background (sample without specific antibodies)	11.80
3	Control (sample with normal plasma)	11.78
4	Experiment (sample with plasma from a special experimental group)	to 49.8

As is evident from the data in Table 3, in the case of immunized animals a substantial increase in milk yield was noted after calving right up to the 60th day of observation for cows immunized with chimeric protein with somatostatin. At the 14th to 30th days this difference reached 21 to 22 % and then stayed at a level of 9 - 13 %; deviations in the data did not exceed 1.5 - 3.0 %. Booster injections of chimeric proteins given in the groups 3 and 5 provided additional increase in milk yield.

Table 3

Indices of milk yields of cows after giving birth in experimental and control groups (%)					
Groups of animals		Days after calving			
		14	30	60	90
1.	Control group	100	100	100	100
2.	Cows immunized three times before calving (carrier - CAT) + +	121	122	106	105
3.	Same with an additional injection on 50th day after calving	121	122	111	121
4.	Cows immunized three times before calving (carrier - DHFR) + + +	109	121	108	107
5.	Same with an additional injection on 50th day after calving	120	121	111	122

+ + Preparations of protein $c(Sp)_4S$ and $c(Sp)_8S$ were used.

+ + + Similar results were also obtained with immunization of milk cows with preparations $c(Sp)_4S$.

As is evident from the data in Table 3, a substantial increase in the milk yield is observed in the case of three-time immunization on the 10th to 20th day after giving birth. This does not depend on the type of protein-carrier (CAT or DHFR) used. The maximum milk yield is observed up to the 30th to 40th day, and then the yield falls to 11 % on the 60th to 90th day, but this reduction can be prevented by an additional (fourth) injection on the 50th day after calving.

8b. Daily weight gains for calves born by immunized cows with additional immunization of the calves after birth.

In these experiments the gravid cows prior to giving birth were immunized three times as indicated in 8a, and the daily weight gain of the calves was measured after birth. In one of the groups the calves were not immunized in order to determine the effect of the immunization of the mothers on the calves. In another group the usual three-time immunization of the delivered calves was carried out at 10-day intervals, beginning with the 20th to 30th day after calving.

It is evident from the data presented in Table 4 that immunization of gravid cows who have not yet given birth results in an increase in the daily weight gain of the calves, the maximum value being 120-125 % at the 30th to 40th day after birth. A similar dynamic weight gain is observed if immunization is made with chimeric proteins comprising CAT or DHFR as the carrier. This shows that there is no effect of the protein-carrier on the efficiency of the claimed chimeric proteins in respect of growth and the meat productivity.

Then the daily weight gain fell to 110-111 % at the 50th to 60th day, but the usual three-time immunization of calves, beginning with the 20th to 30th day after birth, not only prevented this reduction but also caused an additional increase in meat productivity to 135-137 % with respect to the control.

Thus, the stimulating effects due to immunization of the dams during the gravity period and immunization of calves can be summed ensuring maximum increase in the daily weight gains.

Table 4

Indices of weight gains of calves born from immunized cows and subjected to additional immunization after birth (% in respect of control)				
Days after	Immunization of pregnant cows*		Additional immunization of calves delivered by immunized cows	
	protein-carrier		protein-carrier	
	CAT	DGFR	CAT	DGFR
10	112	111	112	111
20	120	120	120	121
30	125	124	125	124
40	120	118	128	126
50	111	110	130	131
60	110	110	135	137

* Three-times immunization of calves on 20th to 30th day after birth with 10-days intervals.

8c. Productivity of swine

Sows of the large white breed receive injections with a preparation comprising a chimeric protein suspended in Freund's incomplete adjuvant approximately 50 days before giving birth. The dose is 50 μg per kg of the weight of the animal, by intramuscular injection in the region of the neck.

Immunization of the sow is conducted three times at intervals of two weeks. The immunized animals do not exhibit manifestations indicating toxicity of the preparation, including disturbances of the reproducing functions (abortions, still-births, etc.). The indices of piglet weight in the experimental group are 1.5 times greater than in the control group (Table 5). In order to maintain a greater meat productivity in piglets delivered by immunized sows, the piglets were immunized three times in accordance with the aforementioned schedule (with two-week intervals and a dose of 50 μg of chimeric protein per kg of animal weight) (Table 6).

Table 5

Indices of the productivity of sows in experimental and control groups		
	Group of animals	Average weight of piglet 15 days after birth
1.	Control (non-immunized animals)	5.10 ± 0.18
2.	Experimental group (immunized with chimeric-protein somatostatin-CAT)	7.73 ± 0.43

Table 6

Indices of meat productivity in piglets under different modes of anti-somatostatin immunization (%)*				
Days after birth	Non-immunized pregnant sows		Immunized pregnant sows	
	Non-immunized piglets	Immunized piglets	Non-immunized piglets	Immunized piglets
30	100	101	112	111
60	100	105	108	115
90	100	106	103	117
120	100	107	102	118

* Percentage of weight increase in relation to figures in the first (control) column: non-immunized pregnant sows and non-immunized piglets taken as 100 %.

Example 9

Factors of stimulation of the productivity of animals subjected to anti-somatostatin treatment.

Table 7

Increase in milk yields and weight gains in the case of immunization with somatostatin-comprising chimeric proteins and protein carriers

Type of product*	Change in % after immunization with:		
	CAT-somatostatin chimera	protein-carrier CAT	DGFR
Milk yield (cows)	120.0	115.0	116.0
Weight gain (piglets)	107.0	102.0	103.0
Weight gain (calves)	130.0	115.0	118.0

* Conditions of immunization with chimeric CAT-somatostatin or native CAT, as in Examples 1 and 2; results in % relative to a non-immunized control group. Results of analysis on the 50th day after onset of immunization.

As is evident from the data of Table 7, together with a substantial increase in the meat and milk productivity of animals when they are immunized with chimeric protein with somatostatin, a sufficiently effective and statistically reliable increase in milk yield and weight gain is also evident when immunization is carried out with a protein-carrier. This stimulating effect may be related to the stimulation of the life activity and productivity of animals under the effect of the immunization procedure itself, independent of the character of the immunogen, which has been noted a number of times in scientific literature. However, in the majority of these studies it was not individual preparations of purified proteins that were used, but rather different kinds of polyantigenic complexes of unidentified composition from cell extracts and biological liquids to mixtures of organic substances of the fluvial mud type, which precludes the possibility of an unambiguous interpretation of the obtained results (Konishev, V.A., 1976, Chemical nature and systematization of substances regulating the process of growth of animal tissue. Successes of modern biology, 81, 2, 258-273). Thus, the final effect of increasing the productivity of farm animals after immunization with

somatostatin-comprising chimeric protein includes the result of the specific effect of anti-somatostatin response and the unspecific effect of immunization with a carrier, wherein immunization with a protein-carrier, in spite of the lower stimulating effect, may also be used independently in practice. At the same time, contrary to somatostatin-containing chimeric proteins, carrier proteins can influence meat or milk productivity only directly in the immunized animals themselves and not in their progeny, when pregnant females were immunized (data not shown), and stimulating effects caused by carrier proteins are observed for a shorter period of time as compared with somatostatin-comprising chimeras.

Deposition of Biological Materials

Strain E. coli MKD3207 comprising the plasmid pC$(Sp)_4$S has been deposited at the Collection of Industrial Microorganisms, Moscow, under accession number VKPM-B-6519.

Claims

1. Chimeric polypeptides having the immunogenicity of somatostatin, including the amino acid sequence of somatostatin-14 and a protein carrier, wherein the sequence of somatostatin-14 is coupled to the 3'-end of the protein carrier through a spacer $(Sp)_n$, where Sp comprises or consists of an alkaline amino acid and an amino acid providing a rigid β-structure, preferably Lys-Pro or Arg-Pro, and n designates the number of blocks in the spacer.

2. The polypeptide of claim 1, wherein the value of n is from 1 to 8.

3. The polypeptides of claims 1 and/or 2, wherein the protein carrier is chloramphenicol acetyl transferase.

4. The polypeptides of claim 3, wherein the chloramphenicol acetyl transferase comprises a deletion of 10 amino acids at the C-terminus.

5. The polypeptide of claims 1 and/or 2, wherein the protein carrier is dihydrofolate reductase.

6. The polypeptide of one or several of claims 1 to 5, which is water insoluble.

7. Recombinant DNA molecules coding for the chimeric polypeptides of one of claims 1 to 6.

8. Recombinant plasmid DNAs coding for the chimeric proteins of one of claims 1 to 6, wherein a sequence coding for the chimeric protein is operatively linked to a regulatory region controlling the expression of the chimeric protein.

9. The recombinant plasmid DNAs of claim 8, wherein constitutive and inducible promoters are used as the regulatory region.

10. The recombinant plasmid DNAs of claims 9 and/or 10, wherein the promoters are selected from lac-T5, Trp, Tac and CAT.

11. The recombinant plasmid DNAs of claim 10, which is selected from plasmids of series pTACAP4S, pT5CAP4S, pT5DAP4S or plasmids of series pC$(Sp)_n$S.

12. Microorganism cells, in particular bacterial cells, preferably E. coli cells, transformed with the recombinant plasmid DNA of one of claims 8 to 11.

13. E. coli cells of claim 12 transformed with a recombinant plasmid DNA of claim 11.

14. E. coli cells of claims 12 and/or 13 which are the E. coli strain MKD3207 comprising the plasmid pC-$(Sp)_4$S deposited under the accession number VKPM-B-6519 at the All-Russian Collection of Industrial Microorganisms, Moscow.

15. Immunogenic compositions, comprising one or several chimeric polypeptides according to claims 1 to 6 in an effective amount, together with a pharmaceutically suitable carrier, excipient and/or adjuvant.

16. The compositions of claim 15, wherein the adjuvant is Freund's incomplete adjuvant.

17. The compositions of claims 15 and/or 16, wherein the weight ratio of purified chimeric somatostatin-comprising protein to the incomplete Freund's adjuvant is from 1:0.05 to 1:10 and preferably 1:1 to 1.2.

18. A method for preparing the chimeric immunogenic somatostatin-containing proteins of claims 1 to 6, comprising cultivating cells of microorganisms transformed with the recombinant plasmid DNA of one of claims 8 to 11 under conditions suitable for expressing these proteins, and subsequently purifying the product of interest.

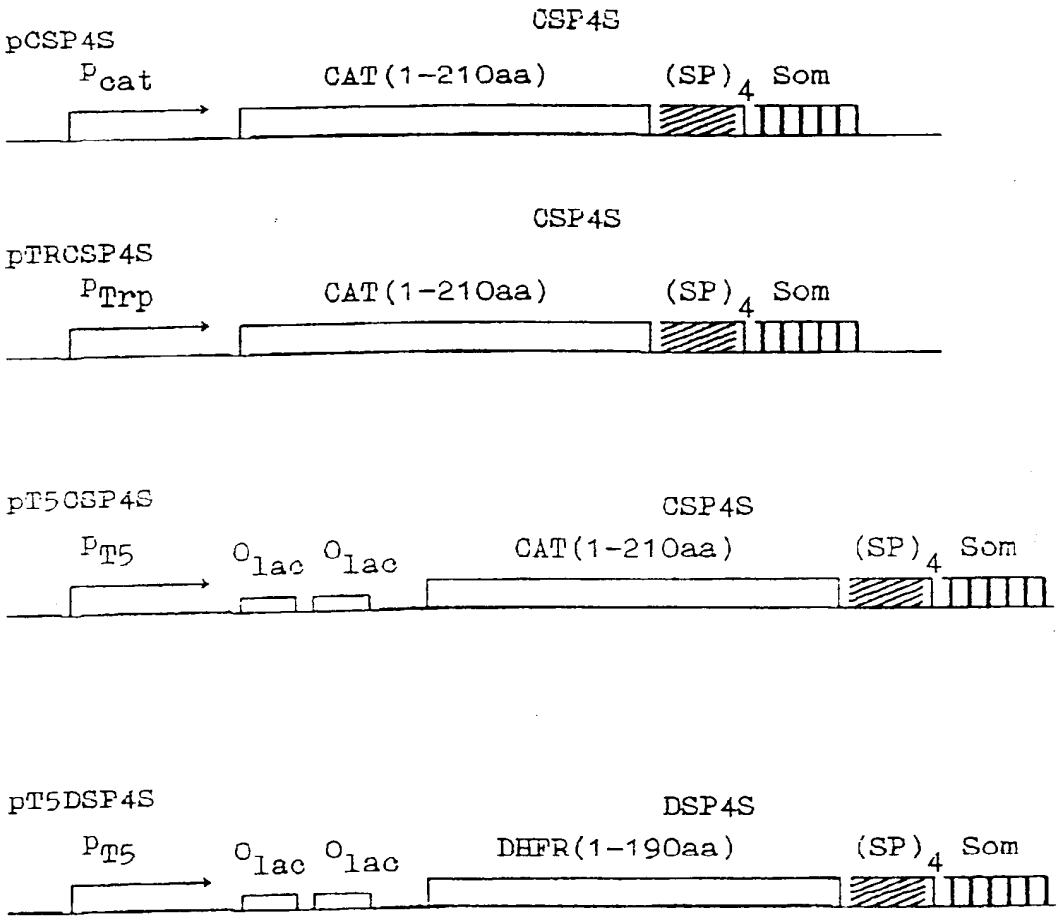
19. The method of claim 18, wherein the cells are E. coli cells, preferably E. coli cells of claims 13 or 14.

20. A method for increasing the productivity and efficacy of farm animals, comprising the administration of an effective amount of the immunogenic composition of one of claims 15 to 17.

21. The method of claim 20, wherein the composition is administered intramuscularly or subcutaneously, the number of injections preferably being from 3 to 5, and the first three injections preferably being carried out at intervals of 10 to 14 days.

22. The method of claims 20 and/or 21, wherein gravid dams are additionally immunized.

23. The method of claims 2o to 22, wherein the animals are cows or pigs.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CSP4S
P_{T5}
O_{lac} O_{lac}
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FIG.1

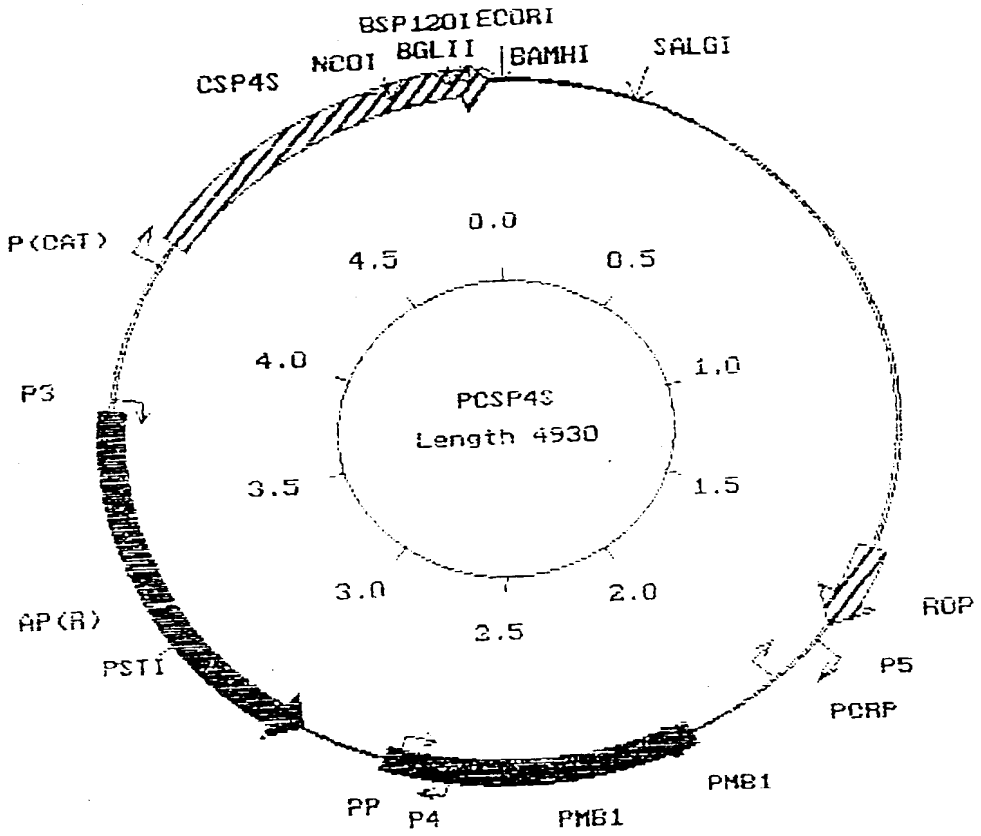

FIG. 2

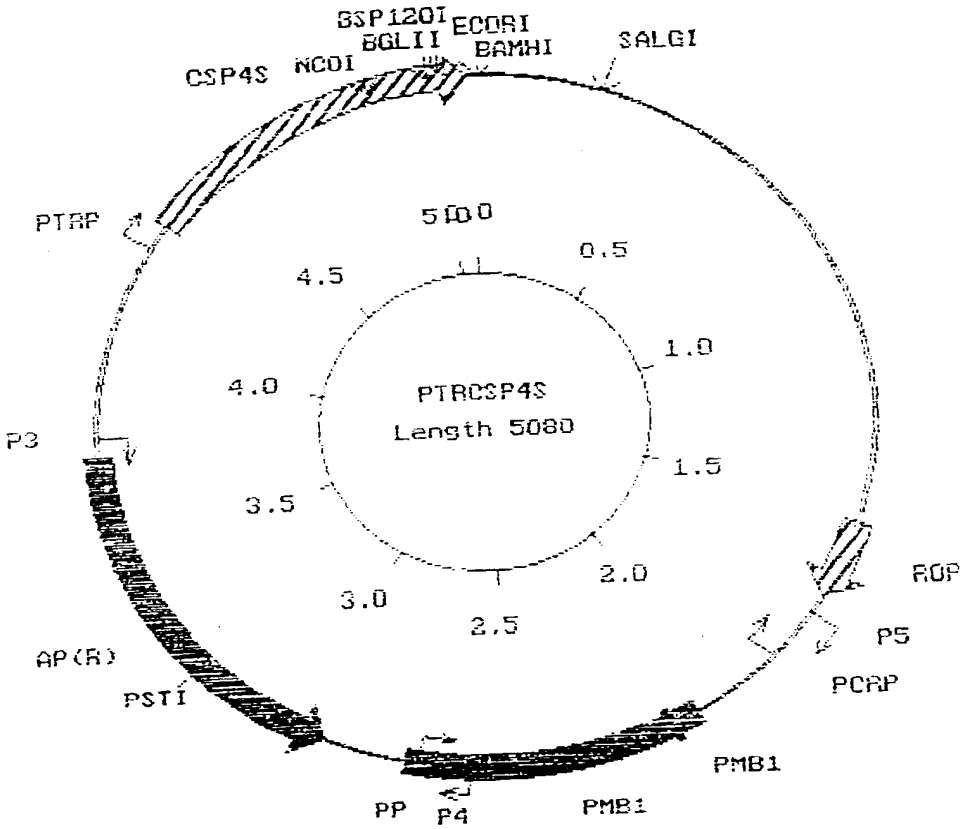
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FIG.3

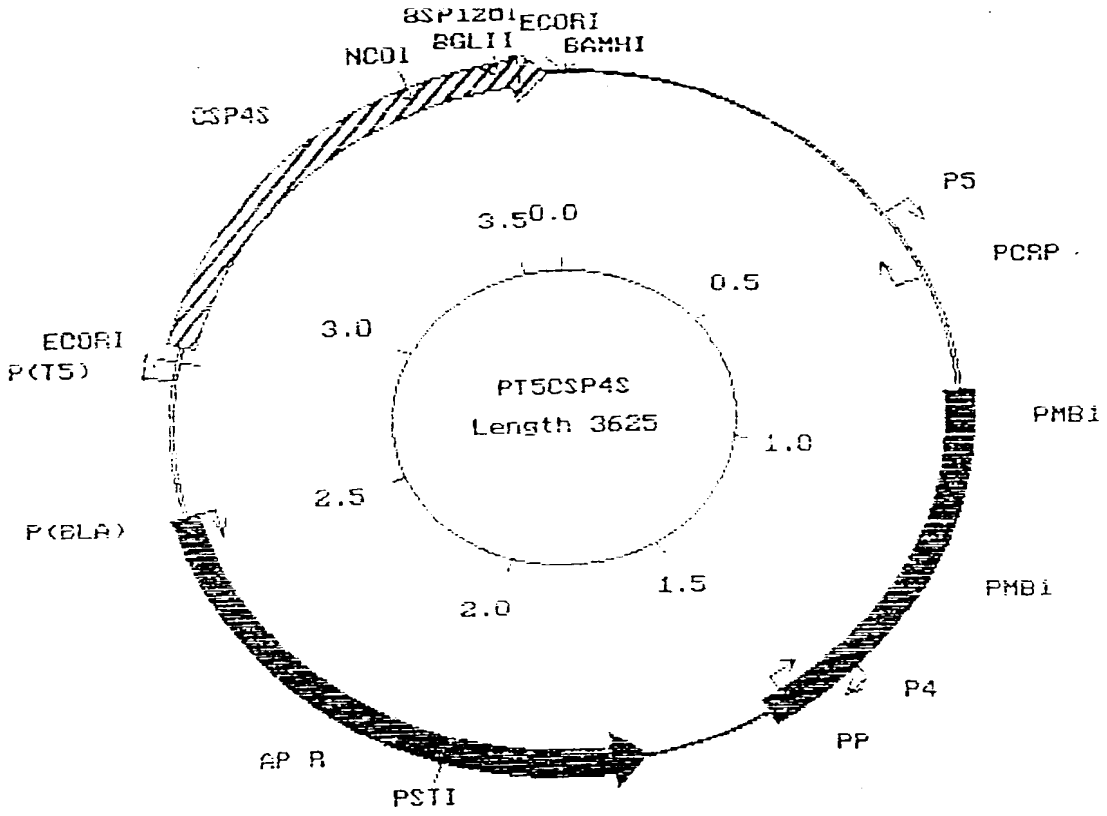
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FIG.4

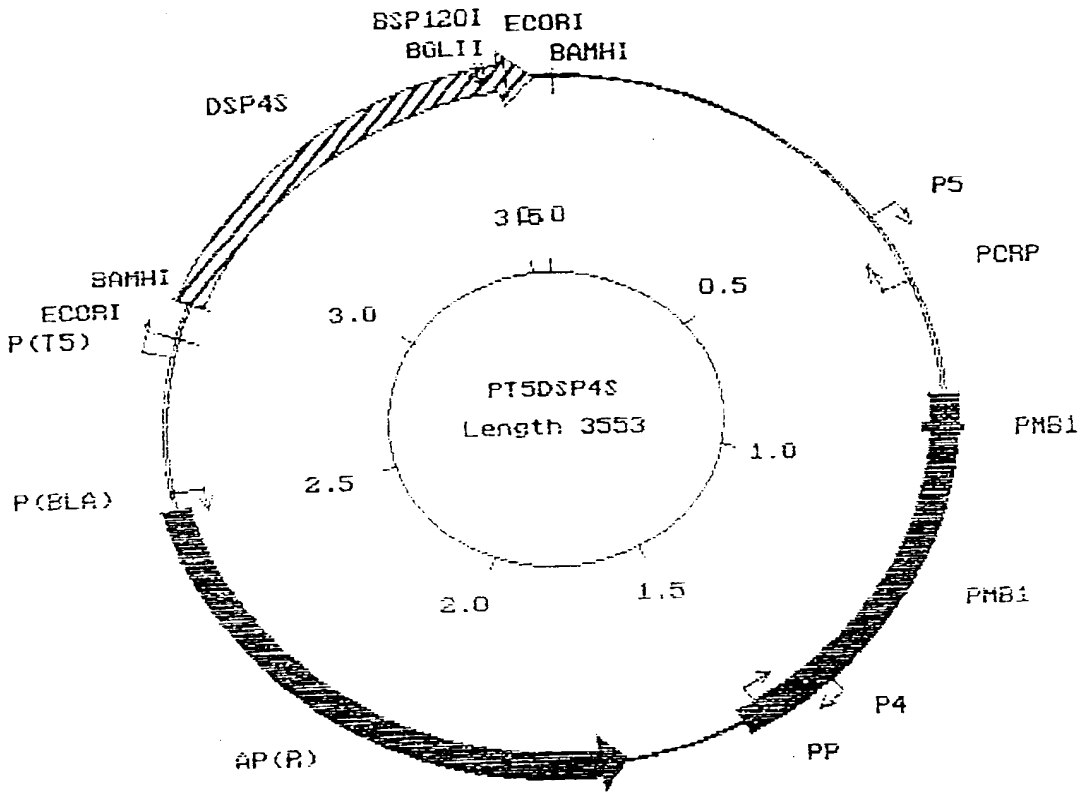

FIG.5

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(54) Chimeric somatostatin containing protein and coding DNA, immunogenic compositions and method for increasing farm animal productivity

(57) Chimeric somatostatins are provided comprising proteins including a protein-carrier to which a spacer sequence $(Sp)_n$ is coupled through ancillary linker-adaptor sequences. The spacer sequence consists of an alkaline amino acid and an amino acid ensuring a rigid β-structure, in particular Arg-Pro or Lys-Pro, coupled to a synthetic sequence encoding somatostatin-14 and a stop-codon.

DNAs coding for chimeric somatostatins comprising proteins and recombinant plasmid DNAs ensuring their expression in microorganism cells, and a method for isolation, purification and refolding of chimeric proteins are also proposed.

Immunogenic compositions are described comprising chimeric proteins as active agent to improve the productivity of farm animals.

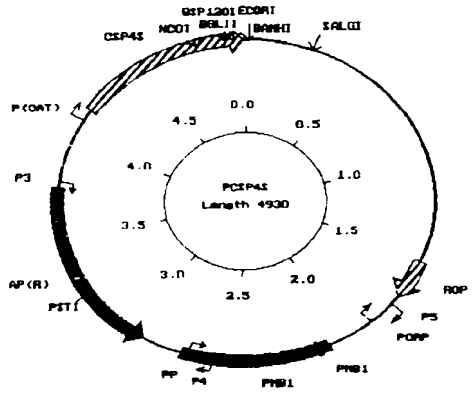

FIG.2

EP 0 645 454 A3

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number EP 94 10 9638

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
A	EP-A-0 001 930 (GENENTECH) 16 May 1979 * the whole document * ---	1-23	C12N15/62 C12N15/16 C12N1/21
A	EP-A-0 270 321 (THE UNIVERSITY OF BRITISH COLUMBIA) 8 June 1988 * the whole document * ---	20-23	C07K14/655 A01K67/02 A61K38/31 A61K47/48
A	EP-A-0 219 106 (F. HOFFMANN-LA ROCHE & CO.) 22 April 1987 * page 4, line 30 - page 5, line 2; example 3 * ---	3-5	C12N9/06 //(C12N1/21, C12R1:19)
A	WO-A-91 10910 (BOEHRINGER MANNHEIM GMBH) 25 July 1991 * page 6, line 9 - 29 and page 9, line 10 - 19 * -----	1-19	

TECHNICAL FIELDS SEARCHED (Int.Cl.5)

C12N
C07K
A61K
A01K

INCOMPLETE SEARCH

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims

Claims searched completely :

Claims searched incompletely :

Claims not searched :

Reason for the limitation of the search:

see sheet C

Place of search	Date of completion of the search	Examiner
THE HAGUE	17 October 1996	Mandl, B

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C07)

EP 94 10 9638

-C-

Remark: Although claims 20-23 are directed to a method of treatment of the human/animal body (Art. 52(4) EPC) the search has been carried out and based on the alleged effects of the compound/composition